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ARTICLE

A transcription-activating polymorphism in the *ACHE* promoter associated with acute sensitivity to anti-acetylcholinesterases

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Hypersensitivity to acetylcholinesterase inhibitors (anti-AChEs) causes severe nervous system symptoms under low dose exposure. In search of direct genetic origin(s) for this sensitivity, we studied six regions in the extended 22 kb promoter of the *ACHE* gene in individuals who presented adverse responses to anti-AChEs and in randomly chosen controls. Two contiguous mutations, a T→A substitution, disrupting a putative glucocorticoid response element, and a 4-bp deletion, abolishing one of two adjacent HNF3 binding sites, were identified 17 kb upstream of the transcription start site. Allele frequencies for these mutations were 0.006 and 0.012, respectively, in 333 individuals of various ethnic origins, with a strong linkage between the deletion and the biochemically neutral H322N mutation in the coding region of *ACHE*. Heterozygous carriers of the deletion included a proband who presented with acute hypersensitivity to the anti-AChE pyridostigmine and another with unexplained excessive vomiting during a fourth pregnancy following three spontaneous abortions. Electromobility shift assays, transfection studies and measurements of AChE levels in immortalized lymphocytes as well as in peripheral blood from both carriers and non-carriers, revealed functional relevance for this mutation both *in vitro* and *in vivo* and showed it to increase AChE expression, probably by alleviating competition between the two hepatocyte nuclear factor 3 binding sites. Moreover, AChE-overexpressing transgenic mice, unlike normal FVB/N mice, displayed anti-AChE hypersensitivity and failed to transcriptionally induce AChE production following exposure to anti-AChEs. Our findings point to promoter polymorphism(s) in the *ACHE* gene as the dominant susceptibility factor(s) for adverse responses to exposure or to treatment with anti-AChEs.

INTRODUCTION

Chemical hypersensitivity to xenobiotics causes adverse responses to normally subacute levels of a specific chemical, or a group of chemicals. Affected individuals may suffer from exaggerated immune response manifested as inflammation of epithelial and mucosal tissues (1,2). Alternatively, they may present altered capacity for scavenging, modifying or degrading a relevant chemical (3). The aberrantly processed chemical may cause toxicological stress in target tissues, with symptoms that vary in nature and timing according to the tissue, type of exposure and the permeability of the chemical. Mutations leading to such aberrant chemical metabolism were identified largely within coding regions, thus affecting detoxifying protein properties. However, impaired transcriptional activation of genes responsible for detoxification, due to mutations in their regulatory sequences, may be an equally important cause of chemical hypersensitivity. For example, the metal-chelating metallothioneins (4) and some members of the cytochrome P450 chemical-modifying enzyme family (5) respond to exposure to xenobiotics by transcriptional activation which increases protection. Impaired transcriptional activation due to promoter polymorphisms in such genes would hence cause chemical hypersensitivity.

Organophosphate and carbamate acetylcholinesterase inhibitors (anti-AChEs) are often implicated in chemical hypersensitivity (6,7). These agents impair neurotransmission (8,9)

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and interact with both AChE and its serum homolog, butyrylcholinesterase (BuChE). Their toxicity has led recently to limitation of their use in the USA as agricultural or home-use insecticides (7,8,10). However, agricultural anti-AChEs are prolifically used in developing countries. Other anti-AChEs are employed as Alzheimer's disease drugs (11) or as prophylactic agents under anticipation of chemical warfare (12). Certain cases of anti-AChE hypersensitivity were attributed to decreased scavenging capacity in carriers of the mutant 'atypical' (6) or 'silent' (13) BuChE variants or to polymorphisms in the paraoxonase gene, *PON1* (14,15), encoding an organophosphate-hydrolyzing enzyme. However, many cases appear to have another, yet undefined origin(s) (16). Previous studies have shown that anti-AChEs promote overproduction of the *readthrough* AChE splice variant (AChE-R) in the mouse brain (17). This induction of AChE production, and the consequent increase in scavenging capacity, confer short-term protection during exposure to such chemicals (18). Conversely, we postulated that an impaired ability for such an induction would be associated with hypersensitivity to anti-AChEs. Impaired transcriptional response to chemical stressors may be due to deficient association with specific transcription factors. Functional polymorphisms affecting chemical hypersensitivity to anti-AChEs are therefore likely to be found close to consensus motifs for stress-associated transcription factors, e.g. the glucocorticoid receptor (GR) (19) or hepatocyte nuclear factor 3 (HNF3) (20).

Here, we describe the identification of two adjacent mutations in a distal upstream enhancer domain of the human (h)*ACHE* gene. One of the mutations, identified in a woman who presented with acute hypersensitivity to the anti-AChE pyridostigmine, was found to constitutively increase AChE expression by abolishing one of two adjacent HNF3 binding sites; the other impairs a GR binding site. Increased sensitivity and impaired transcriptional response to anti-AChEs were also observed in transgenic mice overexpressing hAChE. Moreover, these mice presented increased expression of HNF3β in target tissues. Altogether, our findings imply an association of *ACHE* promoter polymorphism(s) with anti-AChE hypersensitivity by way of a mechanism that probably involves both early modulators such as the HNF3β transcription factor and the downstream responding *ACHE* gene.

RESULTS

Genotyping and sequencing of promoter regions in the *ACHE* locus

Genomic DNA from 103 subjects, including several individuals who suffered cholinergic symptoms under anti-cholinesterase exposure, was subjected to length polymorphism analysis at each of the six regions detailed in Materials and Methods. This analysis identified a 4-bp deletion in region I of the *ACHE* promoter in three heterozygous carriers, including proband I, her mother and proband II. Region I, rich in consensus binding sequences for various transcription factors, includes two sites, 19 bp apart, for binding HNF3β or HNF5/HNF3α. The more upstream of these sites partially overlaps a glucocorticoid response element (GRE) half-palindromic site (Fig. 1B, top) and is abolished by the newly identified deletion (Fig. 1B, bottom). Further screening of region I in 230

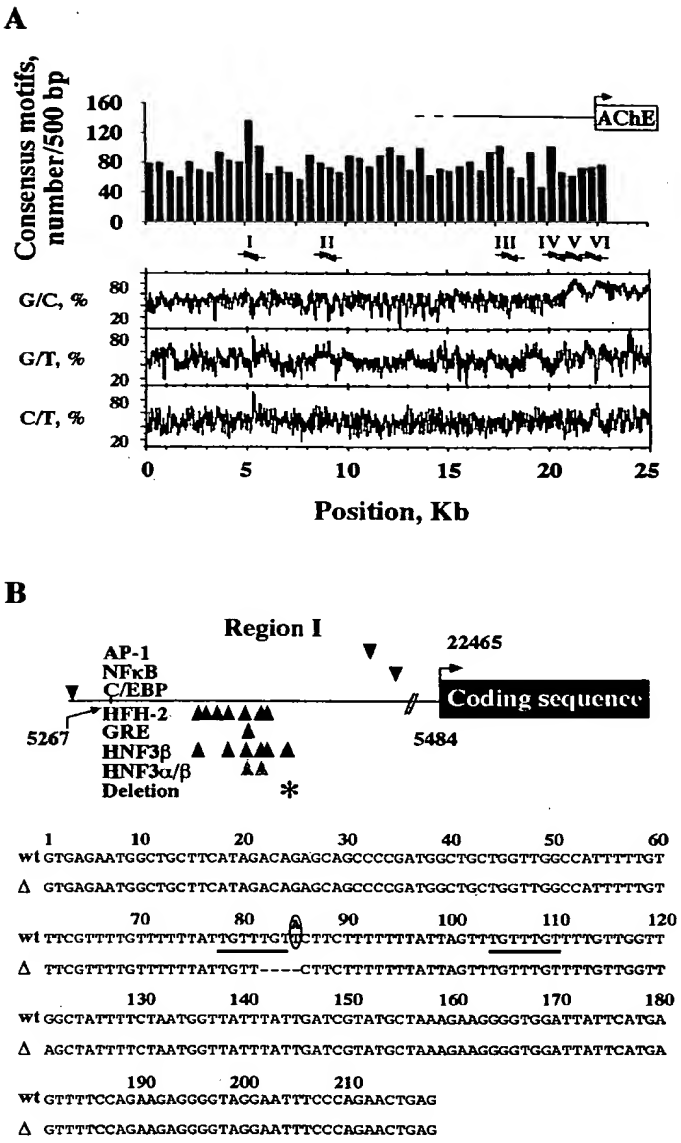

Figure 1. *ACHE* promoter polymorphism in the hypersensitive proband I. (A) Selecting domains prone to effective polymorphism in the h*ACHE* upstream region. (Top) Density of consensus motifs. Shown are cumulative numbers of consensus motifs in 500 bp regions along the AF002993 cosmid reverse DNA sequence. Arrow above represents the *ACHE* transcription start site (nt 22465 in the cosmid sequence; 37,38). (Bottom) Nucleotide pair patterns. Shown are percentages of the noted nucleotide pairs counted in 60 bp windows and 3 nt shifts along the AF002993 DNA. Peaks and troughs represent homogeneous sequences; arrow-delineated Roman numerals represent approximate positions of primer pairs designed to amplify the regions of interest. Note the high number of consensus motifs located in region I. (B) Characteristics of the polymorphic region I. (Top) Consensus binding sites for transcription factors in region I. Presented (triangles) are approximate positions within region I of binding sites for the transcription factors designated on the left. Sites with complete consensus sequences as well as the GRE half-palindromic site (42), TGTTCT, were located using FindPatterns of the GCG software package and the MatInspector program (34). Gray triangles represent consensus sequences known to bind either HNF3α or HNF3β; the asterisk designates the mutated binding site. The first and last nucleotides of region I as well as the transcription start site are marked. (Bottom) Region I sequence. Presented is the normal region I sequence (wt; the T/A substitution is circled) aligned with the mutant sequence allele carrying the 4-bp deletion (Δ). Nucleotide 1 is 5267 in the AF002993 cosmid reverse sequence. The two HNF3 consensus binding sites are underlined.

Table 1. Allele frequencies of cholinesterases gene mutations

Mutation (position[a])	Allele frequency	Sample size	Genotype	Additional linked *ACHE* mutations
ACHE Δ4 (–17116)	0.012	333	all heterozygous	5× H322/N322[d]
ACHE T→A (–17113)	0.006	333	all heterozygous	none
BCHE	0.025	177[c]	7× heterozygous,	ND
D70G[b]			1× homozygous	

Mutation detection was achieved by length polymorphism analysis (for ACHE Δ4) or by PCR and subsequent sequencing (for *ACHE* T→A, *ACHE* H322N and the *BCHE* D70G mutation).
[a]Positions are relative to *ACHE* transcription start site.
[b]The mutation responsible for the 'atypical' BuChE variant.
[c]This is a subset of the sample used for *ACHE* mutation screening.
[d]The biochemically neutral H322N mutation is responsible for the Yt^b blood group. Screening was performed in six carriers of *ACHE* Δ4, three carriers of *ACHE* T→A and 16 non-carriers.
ND, not determined.

additional individuals identified a second mutation, a T→A substitution, which impairs the GRE (Fig. 1B, bottom), and established the allele frequencies of the deletion and the substitution as 0.012 and 0.006, respectively (Table 1), in conformity with a Hardy–Weinberg distribution. Although one carrier of the deletion was hospitalized for multi-infarcts, no increase was detected in the prevalence of any of these mutations in 100 patients hospitalized for infarcts. Carriers of both mutations, who were of various ethnic origins, were additionally screened for the H322N mutation in *ACHE* (21). Five of the six screened deletion carriers were found to be heterozygous for this mutation as well (Table 1). This compares with two heterozygous individuals out of 16 screened non-carriers. No linkage was found between the T→A substitution and the H322N mutation.

Of the total 333 individuals, 177 were also screened for the *BCHE* 'atypical' allele. The allele frequency of 0.025 determined for this sample (Table 1) agrees with the reported frequency range of 0.015–0.05 for 'atypical' *BCHE* in different ethnic groups of the Israeli population (21). In the group currently being analyzed, none of the individuals directed to us due to suspected anti-AChE sensitivity, nor the carriers of the *ACHE* promoter mutations, carried the *BCHE* 'atypical' allele.

Increased basal levels of blood AChE in carriers of the 4-bp deletion

Two carriers of the deletion showed symptoms that may be associated with cholinergic excitation (see Materials and Methods). Proband III displayed gastrointestinal distress compatible with peripheral nervous system (PNS) excitation, which could be attributed to pesticide exposure in her home vicinity, a crop-growing area. Proband I suffered characteristic acute anti-AChE intoxication in response to a subacute dose of the carbamate anti-AChE pyridostigmine. Several years after this incident, peripheral blood AChE levels were measured in proband I as well as in her parents and were found to be slightly higher than normal in both the proband and her mother (Fig. 2A). No differences were found in serum BuChE activity levels, in BuChE inhibition by succinylcholine or in the *BCHE* gene sequence (data not shown), excluding BuChE abnormalities and its potential involvement in the proband's phenotype. Epstein–Barr virus (EBV)-transformed lymphoblast cell lines were established from the second carrier (proband II) and from his non-carrier brother, both negative for the 'atypical' *BCHE* mutation. These cells showed significantly higher AChE activity levels for the deletion carrier than for his brother (Fig. 2A).

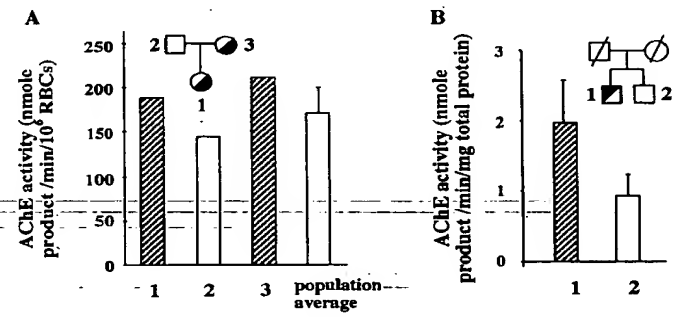

Figure 2. Increased AChE activity levels in blood from carriers of the 4-bp deletion. (A) Red blood cell (RBC) AChE levels in proband I, family and control individuals. Shown is the pedigree of proband I, with the proband and her mother heterozygous for the 4-bp deletion (half-filled circles, see below). Columns present means of triplicate measurements of specific AChE activity in RBC fractions from members of the proband's family. For the control population, presented are mean ± standard deviation (n = 20). (B) AChE levels in EBV-transformed lymphoblasts from a deletion-carrying individual and his non-carrier brother. Presented are AChE activity levels in homogenates of EBV-transformed lymphoblast cell lines established from peripheral blood of proband II, a carrier of the 4-bp deletion and his non-carrier brother, as depicted in the pedigree. Shown are means and standard deviations of AChE levels in seven separate homogenates normalized to total protein measured with the Bio-Rad D_C protein assay kit (Bio-Rad, Hercules, CA).

HNF3β binding assays

The functionality of the two putative HNF3 binding sites in region I was confirmed by electromobility shift assays (EMSAs). Probes containing either one or both of the normal sites all displayed a mobility shift when incubated with cell extracts from HNF3β-overexpressing COS cells (Fig. 3A). An additional supershift caused by rat HNF3β-specific polyclonal antibodies identified the binding activity as HNF3β. In contrast, the mutated upstream site was unable to bind HNF3β, or to compete with the normal upstream site probe for HNF3β binding. Nevertheless, the deletion did not interfere with HNF3β binding to the intact downstream site in a probe representing the mutant allele (Fig. 3A).

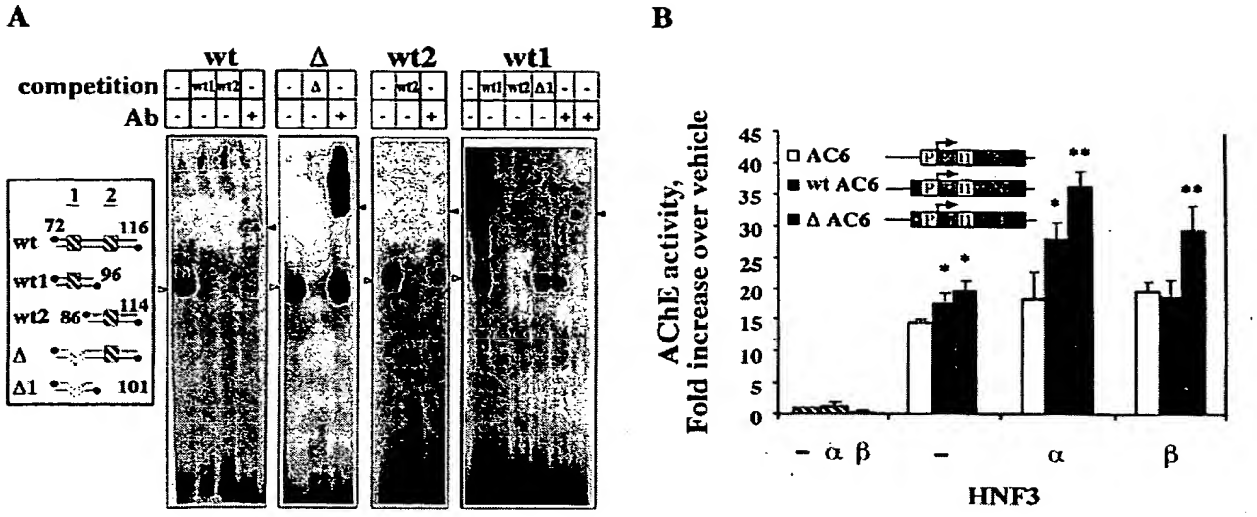

Figure 3. Functional characterization of region I deletion. (A) Gel mobility shift assays reveal differential HNF3β affinities for the two normal and one mutant region I sites. EMSA gel images show shifted probe bands (open triangles), as well as supershifted bands (filled triangles; Ab, antibody in a 1:1000 or 1:500 dilution for wt1 in the two right lanes, respectively). Probes used for each assay are designated above the respective table. (Inset) Presented are the 5′ end-labeled (filled circles) double-stranded oligodeoxynucleotides tested for the binding capacities of the normal and mutant domains in region I. Numbers are as in Figure 1B, bottom. Putative HNF3-binding sites on these probes are boxed and numbered. (B) Differences in transcription activation abilities of the normal and mutant region I sequences. (Inset) Presented are AChE expression constructs used for transfection experiments. Designated are region I normal and variant fragments (dark gray boxes; deletion dotted), the minimal promoter (P), intron 1 (I1), and numbered exons (E). Columns show fold increase values of AChE activities in COS cells transfected with AC6 (open bars), wtAC6 (closed bars) or the ΔAC6 vector (shaded bars), either alone or together with constructs encoding the designated rat transcription factors, both under control of the rat phosphoglycerate kinase-1 promoter. Cross-hatched columns represent transfections with constructs encoding the transcription factors alone. Shown are average specific AChE activities in cell lysates from five transfection experiments as compared with those of cells treated with Lipofectamine alone, in the same set (–). Asterisks mark activities significantly different ($P < 0.01$, Scheffe's test) from those in lysates of cells transfected with AC6 alone. Double asterisks mark an additional significant difference between the wild-type and mutant groups.

The 4-bp deletion increases HNF3β-induced *ACHE* gene expression

To test whether HNF3 binding to the identified sites can modulate transcription from the h*ACHE* gene, we employed the AC6 AChE promoter–reporter DNA constructs to which we added the normal (wtAC6) or deleted (ΔAC6) region I sequence. When transfected into COS cells, each of these three constructs directed h*ACHE* expression, with both versions of region I slightly enhancing the minimal promoter's effect (Fig. 3B). Co-transfection with a construct encoding rat HNF3α increased AChE expression by 50 and 100% for the normal and mutant versions, respectively, compared with the minimal promoter. Rat HNF3β increased AChE expression by 60% for the mutant, but had no effect on the normal allele. Hence, region I includes a functional HNF3-dependent enhancer and the 4-bp deletion increases its effect on AChE expression.

Inherited increases in AChE basal levels impair its anti-AChE-induced overexpression and increase sensitivity to these inhibitors

To test whether AChE overexpression impairs individual responses to subacute doses of anti-AChEs, we used transgenic mice overexpressing human synaptic AChE (22). Exposure to a subacute dose of pyridostigmine caused severe diarrhea in hAChE-transgenics as compared with mild symptoms in control FVB/N mice. Additionally, average survival time under exposure to a lethal dose of diisopropylfluorophosphate (DFP) was 1.9 ± 0.4 min for hAChE-transgenic mice as compared with 5.5 ± 3.3 min for age- and sex-matched control mice ($n = 4$ for each group; $P < 0.05$, Student's t test). Two hours after exposure to a subacute dose of DFP, intestinal AChE was found to be inhibited to 49 ± 38% and 38 ± 7% of its initial activity in transgenic and normal mice, respectively. *In situ* hybridization revealed ~5-fold increases in the stress-associated AChE-R mRNA transcripts in the small intestine (known to express HNF3β) (23) of normal mice. Labeling was localized primarily to the intestinal epithelium, muscularis mucosa and intestinal gland regions where proliferation of epithelial cells takes place (Fig. 4). In contrast to normal mice, transgenics displayed initially higher levels of intestinal AChE-R mRNA, yet showed no significant difference between DFP- and saline-injected groups (Fig. 4).

HNF3β and AChE are co-expressed in hematopoietic progenitors and in the brain

To contribute to anti-AChE responses, HNF3β would be expected to be expressed in AChE-producing cells, which respond to anti-AChE exposure. To examine whether this basic condition is met, HNF3β mRNA was searched for in brain and blood cells by reverse transcriptase–polymerase chain reaction (RT–PCR) and *in situ* hybridization (ISH) analyses. HNF3β production was identified in cortical, cerebellar and hippocampal neurons in the mouse brain (Fig. 5A and data not shown) as well as in AChE-expressing megakaryocytes, lymphocytes and CD34-positive human blood cell progenitors

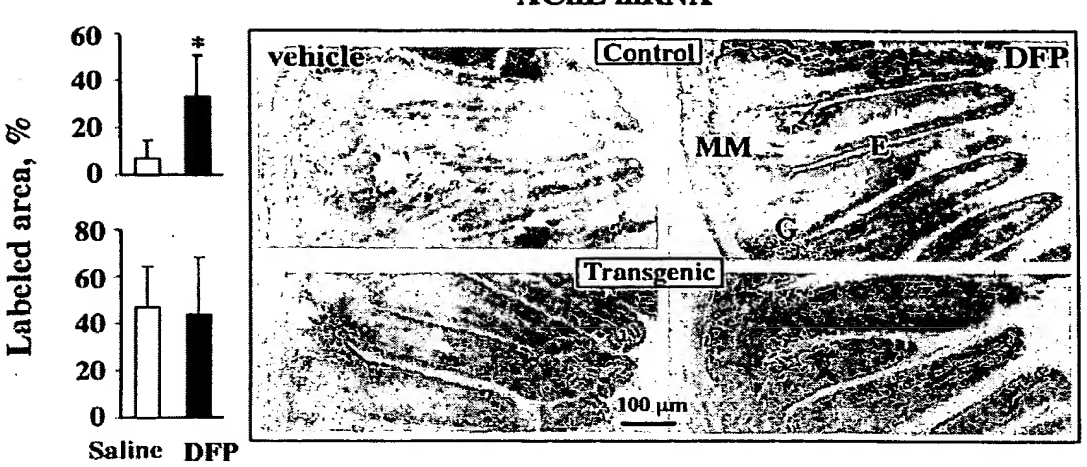

Figure 4. Anti-AChE exposure induces trasncriptional AChE activation in the intestine of normal but not AChE-overexpressing transgenic mice. Presented are representative transverse ileum sections prepared from mice 2 h post-injection (i.p., 1 mg/kg body weight) of DFP or saline. Columns present AChE-R mRNA signal quantified in similar micrographs as percentages of labeled area out of the villus area (means of two to five villi from two to six animals, one to three separate experiments for each animal ± standard deviation). Asterisks denote statistically significant differences ($P < 0.01$, Scheffe's test). Note the drastic increase in AChE-R mRNA levels within the intestinal epithelium (E), the muscularis mucosa (MM) and the intestinal gland (G) regions of DFP-treated normal mice.

(Fig. 5B). Involvement of HNF3β with the impaired transcriptional response to anti-AChE exposure would further predict altered HNF3β expression in AChE-overproducing mice. In keeping with this expectation, brain HNF3β expression, which was barely detectable in normal mice, was conspicuously higher in AChE-overexpressing transgenics (Fig. 5A). Impaired responses to anti-AChE exposure in AChE-overexpressing mammals can therefore include HNF3 contribution in brain, blood and intestinal epithelium alike.

DISCUSSION

We have identified a transcription activating deletion in a distal enhancer domain of the human *ACHE* promoter and demonstrated impairment of the transcriptional activation response to anti-AChE exposure in transgenic mice overproducing AChE. In both mice and humans, AChE overproduction was associated with anti-AChE hypersensitivity. Together, this suggests *ACHE* promoter polymorphisms as novel susceptibility factors for anti-AChE hypersensitivity.

Previously described polymorphisms in the coding regions of *BCHE* (6) and *PON1* (14,15) genes were reported to predispose homozygous carriers to slowly manifested central nervous system (CNS) symptoms of anti-AChE poisoning (6,15). In contrast, the *ACHE* promoter deletion is manifested in dominant and rapidly developing PNS symptoms in heterozygous carriers. The allele frequency of 0.012 defines this deletion as a rare polymorphism in the Israeli population. The other mutation in this region of the *ACHE* promoter, a T→A substitution, was found to be even less abundant, with an allele frequency of 0.006. As carriers of both mutations are of diverse ethnic origin, it is conceivable that these mutations have more than one founder. However, the higher prevalence of the H322N mutation in carriers of the promoter deletion, compared with the reported allele frequency range of 0.06–0.19 in different ethnic groups of the Israeli population (21), suggests a strong linkage between the two mutations.

DNA sequencing, EMSAs and transfection experiments demonstrated that the transcription activation conferred by the 4-bp deletion was due to elimination of a functional binding site for transcription factors of the HNF3 family. HNF3β is known to enhance transcription of several genes through distal enhancer domains (e.g. mouse serum albumin) (24). When included in constructs carrying the normal allele and tranfected into COS cells, the normal HNF3 site hampered transcriptional activation, probably by interfering with HNF3 binding to a second site located 19 bp (~65 Å) downstream. The higher steady-state blood AChE levels in carriers of the mutation and the elevated expression in immortalized lymphoblasts from such a carrier, compared with normal homozygotes, imply that similar activation occurs when the enhancer domain is in its natural context.

That AChE overexpression may hamper anti-AChE responses was shown in transgenic mice, which presented with hypersensitivity to anti-AChEs, accompanied by impairment in the transcriptional activation of AChE production. Such activation was shown previously to occur in the brain, both under inhibition and in response to psychological stress (25). Our current findings suggest that transcriptional AChE overproduction in intestinal endothelium may contribute towards overcoming toxicological stress by offering protection to the peripheral cholinergic systems. While the detailed cause of impairments in the transcriptional activation of AChE remain to be uncovered, one possible factor may be HNF3β which presents increased transcription in the brains of AChE-overexpressing mice.

AChE overproduction was manifested under exposure to both the slowly reversible inhibitor pyridostigmine (17) and the irreversible inhibitor DFP (this report). Such feedback response should be crucial for overcoming exposure to irreversible inhibitors, yet is also important during exposure to slowly reversible

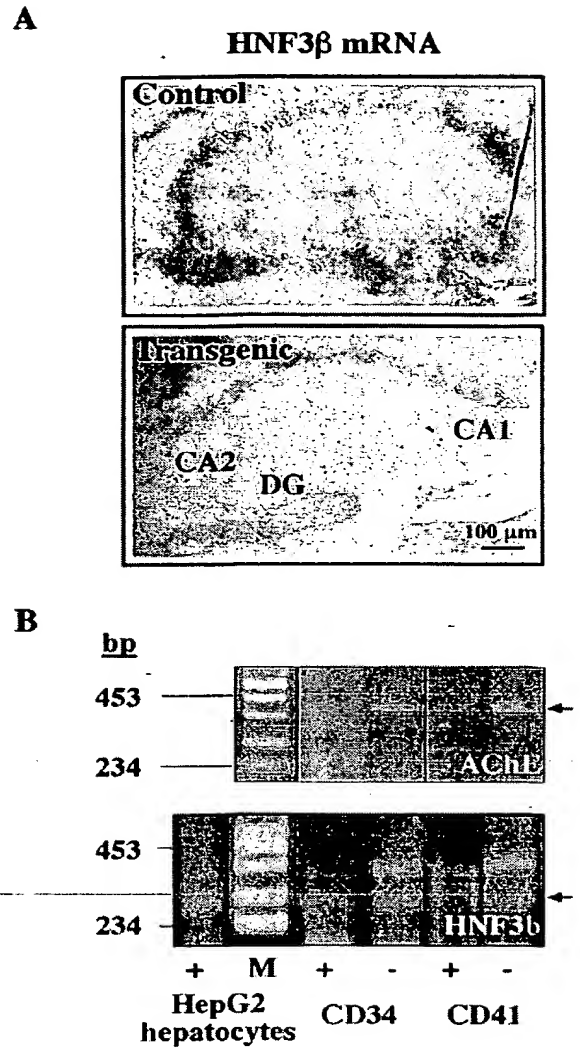

Figure 5. HNF3β is elevated in the brain of hAChE-overexpressing mice and is co-expressed with AChE in diverse human hematopoietic lineages. (A) Hippocampal expression of HNF3β increases in transgenic mice. Representative micrographs of ISH experiments performed on FVB/N mouse sagital brain sections obtained from control and transgenic mice (n = 2 for each group). Shown are the CA1, CA2 and the dentate gyrus (DG) hippocampal structures, known to express AChE (17). Note the increase in HNF3β mRNA, (red signal) in both regions of transgenic mice. (B) Hematopoietic expression. presented are RT–PCR products amplified using primers specific for the domain common to all hAChE splice variants (top) or for rat HNF3β (bottom), from RNA of human hematopoietic cells, sorted by flow cytometry from umbilical cord blood (43). Shown are products from CD34-positive progenitor cells (CD34⁺), CD34-negative fully committed white blood cells and megakaryocytes (CD34–), mature megakaryocytes (CD41⁺) and white blood cells (CD41–). All express AChE and the expected ~300 bp HNF3β product (arrow; also produced in the liver carcinoma HepG2 cell line) accompanied by an ~400 bp unidentified product. M, size marker. No products appeared in control reactions containing no RT (data not shown).

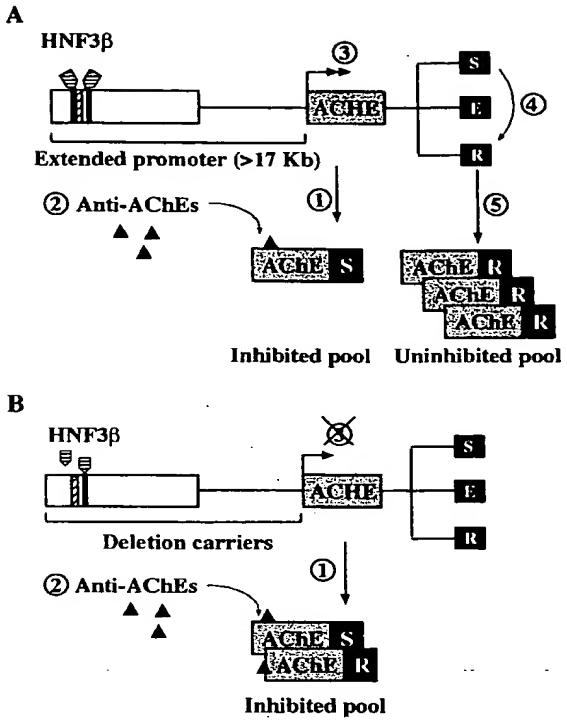

Figure 6. Constitutive AChE overproduction impairs the feedback response to anti-AChEs. (A) Transcriptional AChE overproduction and alternative splicing confer protection by increasing scavenging capacity. The scheme shows the *ACHE* gene and its extended promoter, with the two adjacent HNF3β binding sites (black boxes) and an additional binding site for the glucocorticoid receptor (diagonally hatched box). Numbered steps display the tentative pathway of anti-AChEs responses, as follows. (1) Under normal conditions, the major transcript in both the CNS and PNS is the synaptic variant (AChE-S); hematopoietic cells express preferentially the GPI-anchored AChE-E isoform. (2) Anti-AChEs bind to the active site in the core domain common to all AChE isoforms. This elevates acetylcholine levels, causes cholinergic excitation and thus mimics stress conditions (25). (3) Cholinergic excitation causes enhanced transcription, possibly via the c-*fos* transcription factor, which is thought to activate AChE transcription under stress (17). (4) Newly transcribed AChE mRNA is produced. Alternative splicing preference is for production of AChE-R mRNA instead of AChE-S mRNA. (5) Consequently, a new pool of uninhibited, hyper-sensitive AChE-R molecules accumulates in the tissue, increasing its inhibitor scavenging capacity. (B) Deletion carriers may fail to respond by transcriptional overproduction due to constitutive AChE accumulation. With one HNF3β site missing, the remaining site is more effectively activated by the transcription factor, causing constitutive AChE overproduction (1). This leads to AChE accumulation of which at least a part comprises AChE-R molecules. Anti-AChEs (2) would therefore inhibit preferentially the more sensitive AChE-R variant, leaving some enzyme (possibly AChE-S, or AChE-E in the case of hematopoietic cells) uninhibited. However, the feedback response (3) is impaired. This is apparently crucial for replenishing the enzyme pool to an extent sufficient to suppress acute post-exposure symptoms.

ones. Thus, *de novo* synthesis of a new pool of uninhibited enzyme, to replace the non-functioning pool offers a major pathway for down-regulating inhibitor-induced hyper-excitation. The feedback response to anti-AChE exposure preferentially produces the alternatively spliced stress-associated AChE-R variant (ref. 17 and this report). AChE-R displays significantly higher inhibition constants for several anti-AChEs as compared with the normally produced 'synaptic' AChE-S variant (A. Salmon and H. Soreq, unpublished data). The increased anti-AChE scavenging capacity of AChE-R supports the notion of its involvement with exposure responses, as it would protect the functionally essential synaptic isoform. However, constitutive AChE overproduction such as that occurring in heterozygous carriers of the upstream deletion may prevent sufficient overpro-

duction of these scavenging AChE molecules (Fig. 6). Under exposure, such carriers would therefore lack a fresh pool of uninhibited enzyme, providing a plausible explanation of their apparent hypersensitivity.

HNF3 has been reported to participate in the acute-phase response of the liver to trauma or inflammation (20). In addition to its known expression patterns (23), we found HNF3β to be expressed in hematopoietic cells and hippocampal neurons, both of which are known for their rapid toxicological stress responses (17,26). Our current findings further demonstrate that overexpression of AChE leads to HNF3β overproduction and creates a predisposition to adverse responses to anti-AChE exposure. The greater sensitivity presented by the hAChE-transgenic mice within minutes of exposure to anti-AChEs, suggests a prior, permanent change in the cholinergic system of these mice which is also attributed to AChE overexpression. This change can be caused by the overexpressed HNF3β; alternatively, it may involve non-catalytic functions of AChE, such as its previously reported roles in proliferation and differentiation of various cell types (27). Altogether, this predicts the participation of HNF3-regulated AChE in toxicological stress responses in many tissues, and points to HNF3β as a more general stress-responsive protein than previously realized. AChE regulation by HNF3β may be further influenced by the ubiquitous stress-related GR (19), known to act with HNF3 either synergistically (28) or competitively (29), plausibly affecting the choice between the two HNF3-binding sites in the *ACHE* promoter.

We identified the polymorphic region in the h*ACHE* upstream sequence by combined search for regions of sequence homogeneity rich in clustered transcription factor binding motifs. Similar screens may be useful for future identification of polymorphisms, especially in individuals with chemical hypersensitivity and in genes that are subject to transcriptional activation under chemical exposure. The deletion identified in the *ACHE* promoter appears particularly interesting for screening in individuals suffering from multiple chemical sensitivity (MCS)—a phenomenon still awaiting a clear case definition—which involves multi-organ adverse responses (e.g. gastrointestinal distress and neurological disorders) to normally subacute levels of diverse chemicals (30,31). This syndrome is believed to be caused by neuronal sensitization in specific regions of the CNS limbic system (32). Both stress and anti-AChEs have been shown to elevate AChE expression and to cause cholinergic excitation in the mouse brain (17). As the limbic system is modulated by cholinergic neurons, among others (33), the anti-AChE hypersensitivity presented by proband I suggests a link between increased AChE levels and such MCS-related sensitization.

To conclude, this polymorphism, located 17 kb upstream of the h*ACHE* transcription start site, identifies a new HNF3-binding enhancer domain important for AChE expression. Heterozygosity for the deletion is manifested as constitutive overproduction of AChE; such overproduction, which increases the susceptibility to acute anti-AChE exposures in mice, is likely to be the cause of the hypersensitivity of proband I to pyridostigmine. The proposed link between this mutation and the hypersensitivity points to carriers of this allele as individuals at risk of developing adverse responses under treatment with or exposure to anti-AChEs, which is important in view of the increasing use of anti-AChEs as Alzheimer's disease drugs (11). Moreover, stress- or anti-AChE-induced increases in AChE levels (17) may cause acquired anti-AChE sensitivity, putting at risk a considerably wider group of individuals.(7). This type of chemical hypersensitivity therefore emerges from our study as a complex trait, perhaps involving both early modulators such as transcription factors and downstream responding genes.

MATERIALS AND METHODS

Subjects

A total of 333 individuals were investigated. Of these, 20 were directed to us for investigation of unexplained symptoms with apparent cholinergic involvement, or were family members of such individuals. The majority were randomly selected, with various medical histories and no reported chemical sensitivity. One hundred were older patients hospitalized due to infarcts. The study was approved by the Institutional Review Board of the Herzog Hospital.

Case reports

Proband I, a 30-year-old woman of Ashkenazi Jewish origin and no significant history of adverse drug responses, received a single oral dose of 30 mg pyridostigmine, a dose considered safe, which is given prophylactically under anticipation of chemical warfare (12). Within 1 h, peripheral blood AChE fell to an almost undetectable level, increasingly severe muscle fasciculations developed, accompanied by intense headache, rhinorea, lacrimation and frequent urination. These acute symptoms continued for 3 days, and resolved into a 5-day period of extreme fatigue, muscle weakness and general malaise.

Proband II, a 72-year-old man, was hospitalized due to a multi-infarct dementia, a condition caused by blood flow deprivation during a multi-focal stroke, which damages several brain regions.

Proband III was a 39-year-old woman of Turkish origin with a history of three spontaneous abortions performed under general anesthesia who suffered from excessive unexplained vomiting during a fourth pregnancy.

Selection of screened promoter regions

Promoter regions prone to transcription-modifying polymorphisms were sought in a cosmid clone (GenBank accession no. AF002993) spanning the h*ACHE* gene and ~22 kb of its upstream sequence. Clusters of putative transcription factor binding elements were identified using the MatInspector 2.0 program (34) (core similarity of 1, matrix similarity of 0.85; Fig. 1A, top). Homogeneous sequence regions rich in nucleotide pairs and susceptible to slippage mutation (35) were identified using the Window statistical program of the University of Wisconsin GCG software package (Fig. 1A, bottom). The combined searches yielded six regions of interest: region I, which spans a cluster of putative binding elements (e.g. glucocorticoid response, hepatic and ubiquitous transcription factors such as AP-1) and is G/T-rich; region II, with high C/A content and a 30-nt G/A-rich domain; regions III–V, containing sequence motifs suspected of forming protein-binding DNA secondary structures (36); and region VI, reported to be important for *ACHE* transcription (37,38).

Length polymorphism analysis

Screening involved PCR amplification of assigned genomic DNA regions from peripheral blood lymphocytes, using flanking primer pairs with forward primers 5′-labeled with the fluorophore 6-FAM (Applied Biosystems, Foster City, CA). Electrophoresis (ABI377 automated sequencer, Applied Biosystems) included an internal size marker labeled with a second fluorophore, TAMRA (Applied Biosystems). Fragment length was determined by the ABI GeneScan analysis program. Primers spanned nucleotides 5267–5484 (region I), 9173–9606 (II), 18149–18435 (III), 20709–21029 (IV), 21485–21673 (V) and 22259–22534 (VI), numbered as in the AF002996 reverse sequence.

Genetic screening

DNA samples were subjected to length polymorphism analysis as well as sequencing of region I of the *ACHE* promoter; samples from 177 individuals were also screened for the D70G 'atypical' *BCHE* allele (21) by PCR and subsequent sequencing. All samples positive for mutations in the promoter were also screened for the catalytically neutral H322N polymorphism in the *ACHE* coding region (21).

Cholinesterase assays

AChE and BuChE activity levels were assessed by measuring rates of acetylthiocholine or butyrylthiocholine hydrolysis, respectively, as described previously (6).

Plasmid constructs

Constructs were engineered using amplified DNA fragments from normal (wt) or mutant (Δ) genomic DNA using primers 5267(+) and 5484(–). Ligation upstream of a minimal 600 bp fragment of the h*ACHE* promoter (37) in the AC6 construct yielded wtAC6 and ΔAC6, both encoding human AChE as a reporter.

Cell cultures, transfection and harvesting

COS-1 cells were grown in a humidified chamber in Dulbecco's modified Eagle's medium (Biological Industries, Beit Ha'emek, Israel) supplemented with 10% fetal calf serum (FCS) and 2 mM L-glutamine at 37°C, 5% CO_2. Lymphocytes transformed with EBV were used to create lymphoblast cell lines (39). These were grown similarly to COS-1 with 16% FCS. Transfections of COS cells with 2 μg plasmid DNA per well were carried out using Lipofectamine (Gibco BRL Life Technologies, Bethesda, MD) according to the manufacturer's instructions. Cell homogenates, prepared 2 days post-transfection in phosphate-buffered saline (PBS) containing 1% Triton X-100, were assayed for AChE activity, which is not affected by this detergent. For EMSAs, cells were harvested with cold PBS and homogenized in a buffer containing 10 mM NaH_2PO_4, 400 mM KCl, 10% glycerol, 1 mM dithiothreitol (DTT), 5 μg/ml aprotinin, leupeptin and pepstatin A, and 5 μM NaF. Supernatants, divided into aliquots, were stored at –70°C until use.

Electromobility shift assays

EMSAs were performed using dsDNA probes homologous to restricted parts of region I, essentially as detailed elsewhere (40). Briefly, ~0.5 ng ^{32}P-labeled dsDNA was incubated (2 h on ice) in a total volume of 36 μl of 150 mM KCl, 83 μg/ml poly(dIdC:dIdC), 5 mM DTT, 1 mM EDTA, 5 mM $MgCl_2$, 12% glycerol, 15 mM Tris pH 7.5 and ~20 μg protein from whole cell extracts. Reaction products were electrophoresed in 5% polyacrylamide gels. For competition experiments, a 100-fold molar excess of the corresponding unlabeled probe was used. Pre-incubation of protein extracts (20 min on ice) with anti-HNF3β polyclonal antibodies (1:1000 dilution) was employed for supershift assays.

RT–PCR

Reactions were performed as described elsewhere (37). Primers designed according to rat HNF3β (numbered as in accession no. L09647) were 219(+) and 518(–). Primers for hAChE (numbered as in AF002993) were 25587(+) and 26968(–). Annealing temperatures were 55 and 65°C, respectively. Plus and minus denote forward and reverse primers, respectively.

In situ hybridization

ISH was performed as described elsewhere, using a fluorescent product (41) or the Fast-red-product (Boehringer-Mannheim GmbH, Germany) (17) for labeling. Biotinylated, 2′-*O*-methylated cRNA probes were used, complementary to rat HNF3β mRNA (positions 281–330 in sequence accession no. L09647) or to mouse AChE-R (positions 32–81 in M76540). Fluorescent signal quantification involved one to three sections from separate animals and ISH experiments. Intact villi (excluding the cell-shedding villus tips) were selected using the Adobe Photoshop program. Following determination of signal range, the percentage of labeled areas out of the total selected areas was calculated.

Animal experiments

Mice were injected i.p. with either 1 mg/kg body weight (in ISH experiments) or 7 mg/kg (for survival experiments) of the organophosphate anti-AChE DFP (Sigma, St Louis, MO). Pyridostigmine (0.2 mg/kg; Sigma) served for testing sensitivity to anti-AChEs. Mice were killed 2 h after injection. All experiments were approved by the Committee for Animal Experimentation at the Institute of Life Sciences.

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